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Local and systemic immune responses in pigs intramuscularly injected with an inactivated *Mycoplasma hyopneumoniae* vaccine

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ABSTRACT

The immune response induced by intramuscular administration of a commercial inactivated *Mycoplasma hyopneumonie* whole-cell vaccine (Suvaxyn®MH One) was investigated in conventional *M. hyopneumoniae*-free pigs. The animals were assigned randomly to two groups: non-vaccinated and vaccinated. Pigs in the vaccinated group were injected intramuscularly with the vaccine at 7 days of age, whereas non-vaccinated pigs received physiological saline solution (PBS). Pigs were euthanized and necropsied at 30, 36 and 58 days of age. Blood, bronchoalveolar lavage (BAL) fluid, spleen, lung and bronchial lymph nodes (BLN) were collected. Serum and BAL fluid were tested for the presence of antibodies by ELISA. Monomorphonuclear cells from the peripheral blood and tissues were isolated to quantify the T cell subsets by flow cytometry, and cytokine production by ELIspot and ELISA. Antibodies against *M. hyopneumoniae* were detected in serum of most vaccinated pigs at 30 days of age. *M. hyopneumoniae* specific IgG, IgM and IgA were detected in BAL fluid from vaccinated animals, but not from control animals. Significantly higher numbers of IL-12 secreting cells were observed in the lung at day 58 in the vaccinated than in the non-vaccinated group ($p < 0.05$). The number of IL-10 secreting cells from BLN was also higher in the vaccinated group at day 58 ($p < 0.05$). After restimulation in vitro, lymphocytes from BLN and lungs secreted significantly higher levels of IL-12 in the vaccinated group at day 58. These results show that the vaccine induced both systemic and mucosal cellular and humoral immune responses.

1. Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the primary pathogen of enzootic pneumonia (EP). The disease occurs worldwide and causes important economic losses to the pig industry [1]. Optimization of housing and management procedures combined with vaccination are the most effective tools to control EP [1,2]. The currently used vaccines consist of adjuvanted, inactivated, whole-cell preparations and have been proven to be effective in reducing the clinical symptoms and lung lesions, and in decreasing performance losses associated with *M. hyopneumoniae* infections [1]. They are very frequently used worldwide in commercial pig herds.

The commercial vaccines induce specific antibodies in serum, although no direct correlation has been found between the induction of antibodies and protection against *M. hyopneumoniae* [3]. Some studies suggest that mucosal antibodies and cellular immune responses might be important for the control of this disease [4].

Only in few studies the effects of vaccination on mucosal and cell mediated immunity have been evaluated. Studies on bronchoalveolar lavage (BAL) fluid and lymphocytes isolated from blood evaluated by ELIspot and ELISA showed that a commercial inactivated vaccine induces mucosal and systemic cell mediated immune response. However, the vaccination failed to induce changes in the peripheral $CD4^+$ and $CD8^+$ cells population [4]. In contrast, differences between vaccinated and control pigs in the percentage of these cell populations, were detected in a study performed by

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Kick et al. (2011) [5]. The considerable variations between published reports demonstrate that the exact mechanism of protection induced by inactivated *M. hyopneumoniae* vaccines is not known.

The aim of this study was, therefore, to analyze the systemic as well as mucosal immune responses induced by intramuscular vaccination of pigs with a commercial *M. hyopneumoniae* inactivated whole-cell vaccine that had previously been shown to be effective in reducing disease signs and lung lesions associated with *M. hyopneumoniae* infection [6].

2. Materials and methods

2.1. Experimental design

The study was performed after approval by the Ethical Committee for Animal Experiments of the Faculty of Veterinary Medicine, Ghent University (approval number EC2011/038). Thirty-three cross-bred healthy piglets free of *M. hyopneumoniae* and PRRSV were used in this study. All animals were obtained from a herd that has been free of *M. hyopneumoniae* and PRRSV for more than 15 years based on repeated serological testing and absence of clinical symptoms and pneumonia lesions. At one week of age, blood for serology was collected from all animals. Thereafter, the piglets were randomly allocated to two different groups. At 7 days of age, 17 pigs of the vaccinated group were intramuscularly injected with 2 ml of the commercial *M. hyopneumoniae* vaccine Suvaxyn®MH One, Pfizer Animal Health, and 16 pigs of the control group were intramuscularly injected with 2 ml of phosphate buffered saline (PBS). The piglets were weaned at 24 days of age, transported to the Faculty of Veterinary Medicine, Ghent University, where they were necropsied at 30, 36 and 58 days of age. All piglets were euthanized by deep anesthesia with 0.3 ml/kg of a mixture of Xylazin (Xyl-M 2%®, VMD, Arendonk, Belgium) and Zolazepam and Tiletamine (Zoletil® 100, Virbac, Louvain la Neuve, Belgium), followed by exsanguination. At necropsy, lungs were macroscopically examined for the presence of lesions and blood and tissue samples from the spleen, the bronchial lymph nodes (BLN) and lungs were taken from all pigs. The lungs were removed and bronchoalveolar lavage (BAL) fluid was collected from the right lung. Blood was collected and part of it was used for serology. The other part was mixed with heparin (1:1000) and used for isolation of peripheral blood monomorphonuclear cells (PBMCs).

2.2. Nested and quantitative PCR (qPCR) for detection of M. hyopneumoniae DNA

DNA was extracted from the BAL with the QIAGEN protocol (QIAGEN, DNeasy Blood & Tissue kit, Belgium). For detection of *M. hyopneumoniae* DNA, both a nested and qPCR were performed as described previously [7,8], in order to avoid false negative results.

2.3. Isolation of monomorphonuclear cells from blood and tissues

PBMCs were isolated by density gradient centrifugation on Lymphoprep™ (Axis-Shield, Oslo, Norway). After lysis of erythrocytes in ammonium chloride (74.7%) and subsequent centrifugation, the pelleted cells were washed, resuspended in PBS with 1 mM of EDTA and counted.

After euthanasia, monomorphonuclear cells were isolated from the lungs, the BLN and the spleen. The monomorphonuclear cells were isolated by tearing the tissue apart, followed by lysis of erythrocytes with 0.15 M ammonium chloride. After centrifugation, the pelleted cells were washed, resuspended in leukocyte medium and counted [9].

2.4. Detection of M. hyopneumoniae-specific antibodies

Serum collected from the pigs at the day of vaccination and at necropsy was analyzed for the presence of antibodies against *M. hyopneumoniae* using a blocking ELISA (IDEIA™ *Mycoplasma hyopneumoniae* EIA kit, Oxoid Limited, Hampshire, UK).

To determine the isotype of *M. hyopneumoniae*-specific antibodies in serum, peroxidase labeled goat anti-porcine IgA, IgG and IgM polyclonal antibodies (Bethyl Laboratories, Texas, TX, USA) and *M. hyopneumoniae* antigen coated microtitre plates from the IDEXX Mhyo ELISA (IDEXX, Hoofddorp, The Netherlands) commercial kit were used.

Previous experiments have shown that no reaction can be detected in BAL fluid using plates from the IDEXX kit which was developed for detection of antibodies in serum, so plates were coated with Tween®20 extracted *M. hyopneumoniae* antigens [10]. BAL fluid samples were assayed for IgG, IgM and IgA antibodies against *M. hyopneumoniae* using peroxidase labeled goat anti-porcine IgA, IgG and IgM polyclonal antibodies (Bethyl Laboratories). The BAL fluid was tested undiluted. OD at 450 nm was measured and the mean OD value of the serum from the non-vaccinated animals plus two times the standard deviation was used as cut-off value to determine the number of positive animals in the vaccinated group for each immunoglobulin. Values equal to or higher than the cut-off were considered as positive.

2.5. Determination of T cell populations in different tissues

Flow cytometry was used to quantify the T-cell subset populations ($CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD4^+CD8^+$ and $CD3^+CD4^-CD8^-$) from the PBMCs and monomorphonuclear cells from spleen, the BLN and lungs. Monomorphonuclear cells (1×10^6 cells) were incubated for 20 min on ice with monoclonal antibodies against CD3 (PTT3, IgG1) [11], CD4 (74-12-4, IgG2b) [12] and CD8 (11/295/33, IgG2a) [13] (Becton Dickinson, Erembodegem, Belgium). After the wash steps, the cells were incubated with the appropriate isotype-specific FITC rat anti-mouse IgG1 (BD Pharmingen), AlexaFluor®647 goat anti-mouse IgG2a and R-phycoerythrin goat anti-mouse IgG2b (Invitrogen). Mouse IgG1, IgG2 and IgG2b were used as isotype controls. After each incubation step, cells were washed twice with PBS. Finally, the cells were diluted in 400 μl and analyzed using a FACSCanto flow cytometer (Becton Dickinson Immunocytometry Systems, Erembodegem, Belgium) equipped with two lasers, a 488 nm solid state laser and a 633 nm HeNe laser, and FACSDiva software. For all samples, at least 10,000 cells were counted. All data were corrected for autofluorescence as well as for unspecific bindings using isotype-matched negative controls.

2.6. Enumeration of cytokine secreting cells

The numbers of IFN-γ, IL-10 and IL-12 secreting cells in PBMCs and tissue monomorphonuclear cells were determined by use of an ELIspot assay. Ninety-six well microtitre plates were coated with monoclonal antibodies against porcine IFN-γ (Swine IFN-γ Antibody Pair, Invitrogen, Merelbeke, Belgium), IL-10 (IL-10 Swine Antibody Pair, Invitrogen) and porcine IL12/IL23 p40 (Porcine IL-12/IL-23 p40 DuoSet, R&D Systems, MN, USA). After incubating the plates overnight at 4 °C (IFN-γ, IL-10) or at room temperature (IL-12), they were washed with PBS and blocked with PBS + 1% bovine serum albumin (IFN-γ) or RPMI + 10% fetal calf serum (IL-10 and IL-12) for 1 h at 37 °C (IFN-γ, IL-10) or at room temperature (IL-12), followed by a second wash step. Monomorphonuclear cells (5×10^4/well for IFN-γ, IL-12 and 2×10^4/well for IL-10) in leukocyte medium alone (no stimulation) or with *M. hyopneumoniae* antigen (10 μg/ml) were added to the wells, and plates were incubated for 20 h at 37 °C in 5% CO2. The plates were washed and